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Comparison of Three Earthworm Bioassay Procedures for the Assessment of Environmental Samples Containing Hazardous Wastes

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16. ABSTRACT

Three differenct laboratory earthworm protocols for assessing the potential toxicity of environmental samples were evaluated using Eisenia fetida. The 48-h Contact Test (CT) is a short test and may indicate the presence of water-soluble chemicals. The 14-day Soil Test (ST) is best suited to assess chemical impacts of either known or unknown chemicals in soils. The Giant Axon Conduction Velocity protocol(GACVT) used with either the CT or the ST can detect sublethal neurotoxic effects in shorter periods of time and at lower chemical concentrations than mortality tests. The use of these tests is suggested as an estimate of potential toxicity of environmental soil samples.

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A comparison of three earthworm bioassay procedures for the assessment of environmental samples containing hazardous wastes

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Summary. Three different laboratory earthworm protocols for assessing the potential toxicity of environmental samples were evaluated using *Eisenia fetida*. The 48-h Contact Test (CT) is a short test and may indicate the presence of water-soluble chemicals. The 14-day Soil Test (ST) is best suited to assess chemical impacts of either known or unknown chemicals in soils. The Giant Axon Conduction Velocity protocol (GACVT) used with either the CT or the ST can detect sublethal neurotoxic effects in shorter periods of time and at lower chemical concentrations than mortality tests. The use of these tests is suggested as an estimate of potential toxicity of environmental soil samples.

Key words: Earthworm bioassay – Soil toxicity assessment – Complex mixtures – Hazardous materials

Earthworms have been used in various tests to assess the toxicity of chemicals (EEC 1982; Karnak and Hamelink 1982; Dean-Ross 1983; Roberts and Dorough 1985). Since the publishing of the EEC methods for testing, the 48-h Contact Test (CT) and the 14-day Soil Test (ST) have been generally accepted as standard methods for exposing earthworms in the laboratory (Neuhauser et al. 1985). Comparisons of various methods have been made for laboratory toxicity assessment (Dean-Ross 1982; Fisher 1984; Heimbach 1984). Modifications of the EEC methods have been made in an attempt to improve the ability for predicting the impact of chemicals in field situations. These include mixing the chemical with soil in the laboratory (Ebing et al. 1984) and applying the chemicals in the field (Haque and Ebing 1983).

During the past 3 years the US EPA Corvallis Environmental Research Laboratory (CERL) Hazardous Materials Assessment Team (HMAT) has been evaluating a variety of biological test methods for their utility in identifying the presence of hazardous materials in soils and water (Porcella 1983; Callahan, in press). The primary goal of this overall effort is to identify cost-effective methods for evaluating the potential toxicity of hazardous waste sites.

Three different earthworm protocols have been tested to date: the 14-day Soil Test (ST), the 48-h Contact Test (CT), and the Giant Axon Conduction Velocity Test (GACVT). The CT and the ST are methods proposed by the EEC (1982) whereas the GACVT was developed by Dr. C.D. Drewes at Iowa State University. While the CT and the ST have been used primarily to evaluate the safety of chemicals before release to the environment (Goats and Edwards 1982), Neuhauser et al. (1982) have used them to assess the impact of heavy metals and organic chemicals on soil biota. Drewes and Vining (1984) and Drewes et al. (1984) have used the CT to show that the GACVT for earthworms is a sensitive means of assessing the impact of neurotoxic compounds on earthworms under laboratory conditions. Although the relative toxicities of a limited number of pure chemicals are known from these bioassay protocols

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there are virtually no data available for evaluating the toxic reactions of earthworms to complex mixtures of contaminated environmental samples. The objective of this study is to compare these protocols with respect to their utility for assessing the potential toxicity of contaminated environmental samples to earthworms.

Materials and methods

General. Eisenia fetida (Savigny) was selected as the test species because it is relatively responsive to chemicals (Roberts and Dorough 1984) and is easily reared in the laboratory (Malecki et al. 1982). Specimens used in this study were obtained from cultures maintained at CERL. The cultures were maintained at 20°–25°C under continuous light. Commercial rabbit feed in the form of alfalfa *(Medicago sativa)* pellets, saturated and held for 2 weeks, were used as the food substrate for the worms. The worms were harvested the day of the test and adult (clitellate) specimens were selected at random for testing.

Soil is routinely collected by Field Investigation Teams of the US EPA at sites where toxic and hazardous materials have been spilled or stored. Two sample sites include a Morton Thiokol site and an Olin Chemical Co. site. The soil was shipped to CERL for evaluation and was processed as described by Porcella (1983). Soil samples were air dried before elutriates were prepared from the soil using a 4:1 weight ratio of water to soil. The physical properties of the sample soils were obtained from analysis performed by the Oregon State University Soils Department (Table 1). The pHs of the mixture of artificial soil and the Olin sample were obtained in our laboratory. The elutriate was mixed for 48-h (150 rpm) at constant temperature (20° ± 2°C) in the dark. The mixture was allowed to settle and the liquid decanted and filtered with a 0.45-μm membrane to obtain the elutriate. Samples with high clay content were centrifuged and decanted prior to filtration. The elutriate preparation was used only for the CT. In this protocol a range of concentrations were produced by mixing the elutriate with distilled water. Preliminary screening tests (i.e., range finding) were used to determine the approximate range of the LC_{50} for the elutriates. When necessary, more definitive ranges could be used to determine the specific LC_{50} values. However, for this study only range-finding tests were run for both the Morton Thiokol soil and the Olin Chemical Company soil elutriates because the results indicated very low toxicity.

Contact Test. The CT protocol described by Edwards (1983) and Goats and Edwards (1982) was followed for all elutriate testing. In this protocol, glass vials 8 cm long × 3 cm diameter were lined with Whatman No. 1 filter paper to which 1 ml of the elutriate was added and capped. One worm was added to each vial and the test vials were held on their side at 20°C in the dark. Vials were inspected twice daily to insure that the worm was in contact with the filter paper. After 48-h, worms were recorded as dead if they did not react when gently touched on the anterior end with a probe.

The CTs were performed only at the screening level because of the lack of apparent elutriate toxicity. There were two runs of each site elutriate with ten replications at 100%, 50%, and 0% (distilled water) concentrations. The Olin Chemical Company elutriate showed slight toxicity in one run where five of ten worms died at 100% and none died in the other run at the same concentration.

Soil Test. The ST was modified slightly from the methods described by Edwards (1983) and Goats and Edwards (1982) to accommodate mixing artificial soil with the site soil. The artificial soil was prepared by weighing and mixing ground sphagnum peat (10%), kaolinite (20%), and 70 mesh sand (70%) and adjusting the final pH to 6.0 ± 0.5 with pulverized calcium

Table 1. Physical and chemical properties of Morton Thiokol, Olin, and the artificial soils

	pH (H_2O)	% Organic matter	Cation exchange capacity	Sand	Silt (%)	Clay
Morton Thiokol Co.	7.5	2.9	6.0	72.5	19.6	7.9
Olin Chemical Co.	1.4	2.8	3.1	66.3	30.7	3.0
Artificial soil	5.2	2.8	10.1	78.2	2.8	19.0
Artificial soil/Olin soil mix (w/w)						
10% Olin soil	3.9					
25% Olin soil	3.0					
50% Olin soil	2.2					
75% Olin soil	1.8					

carbonate. The pH adjustment is based on a normal requirement of 1.6 g CaO equivalent per kilogram of mix. Samples from some batches fall outside these limits, e.g. pH of 5.2 for the artificial soil sample (Table 1). The artificial soil was used to make various "concentrations" of site soil by mixing with varying amounts of the air-dried Morton Thiokol and Olin Chemical Co. site soil (dry wt./dry wt). The moisture was adjusted to equal 35% dry wt. of the artificial soil or 75% of field capacity of the site soil in the mixture. The Morton Thiokol sample soil was mixed with artificial soil to produce mixtures for testing at 55%, 45%, 40%, 35%, 30%, 25%, and 0% (control) levels. The Olin Chemical Co. soil was tested for mortality at concentrations of 20%, 12.5%, 10%, 7.5%, 5%, and 0%.

There were ten worms per jar in each of four replications at each level including controls. Each test mixture was placed into 570-ml jars containing 400 g of the moist soil mixture; for less dense mixtures, the jars were filled to within 3 cm of the jar top. The jars were placed in an incubator at 20°C in continuous light and mortality was assessed after 14 days. Since dead *E. fetida* usually decay rapidly in soil, mortality was inferred by failure to recover intact worms after careful search of the soil.

Giant Axon Conduction Velocity Test. The Morton Thiokol and Olin Chemical Co. soils were used to conduct the sublethal GACVT. Morton Thiokol soil was tested at 0%, 15%, 25%, 35%, and 50% site soil by weight. GAVC studies were conducted to evaluate the effects of Olin Chemical Co. soils over various time intervals at 5%, 10%, and 20% concentrations of the original soil sample. This test was conducted to evaluate the use of GACVT protocol for time intervals less than 14 days. The GACVTs were prepared in the same manner as the ST except six petri dishes (100 × 20 mm) were used for each concentration and a single worm was placed in enough soil in each dish to fill the dish (75–85 g). In this way worms could be measured and tracked individually.

Giant Axon Conductivity Velocity Test measurements were made before and after exposure to the soil (Drewes et al. 1984). The GACV varies with several factors including the size and the age of the worm. By measuring the conduction velocity before and after exposure, and using the relative change (rate after/rate before × 100), each worm is used as its own control, thus reducing problems of interanimal variability as a factor in the actual conduction velocity. There were five to six measurements of the conduction velocity of the median nerve made for each worm at each sampling time. The mean conduction velocity is reported for each worm at each concentration for every sampling time. These data were analyzed by analysis of variance (ANOVA) (Steel and Torrie 1960) for significance between the various soil concentrations and time intervals. Duncan's New Multiple Range Test (Kramer 1956; Steel and Torrie 1960) was used to separate the mean values of conduction velocities at the soil concentration for each time interval.

The following precautions were taken to obtain representative measurements of conduction velocities (Drewes et al., in press):

1. Replicate measurements (five to six) were made within the same body region
2. Measurements were made from the first impulse in a train of impulses
3. The site of stimulation was well anterior or posterior to all recording sites
4. Measurements were made only when specimens were oriented in a straight line and in a reasonably elongated state
5. All measurements were made at a constant temperature

The coefficient of variation (CV) for all GACVT measurements was 17% or less for both Morton Thiokol and Olin Chemical Company soil.

The Morton Thiokol soil sample was tested at six concentrations and four replications for each of two runs. To evaluate precision of the tests, each replication was used to estimate an LC_{50}, and the estimated LC_{50} was compared among replications and between runs by chi-square analysis (Steel and Torrie 1960). There were no significant chi-square values among replications or between runs. The same evaluation was made for the three replications and three runs of the Olin Chemical Company soil with the same results; all chi-square values were insignificant.

Values for LC_{50} for all soil tests were estimated using the methods described by Hamilton et al. (1977).

Results and Discussion

The CTs conducted on elutriate samples from both the Morton Thiokol sample and the Olin Chemical Co. soil samples indicated very low toxicity (Table 2). The Morton Thiokol elutriate sample showed no effect whereas the Olin sample had an estimated LC_{50} of approximately 100% (i.e., full strength). The Morton Thiokol sample was suspected of having high concentrations of diphenylamine in addition to unknown polynuclear aromatic hydrocarbons. The CT can be used as a preliminary screen for aquatic

Table 2. Comparative results of soil and elutriate evaluations using lethal and sublethal (GACVT) end points

Sample	End point	LC_{50} (95% CI)	*F* ratio	(tabular *F* value)	Duncan's Multiple Range Test
Morton Thiokol elutriate	Mortality CT	No effect at 100% concentration			
Morton Thiokol Soil	Mortality ST	33.49% (32.36%–34.67%)	34.59	5 ($F = 4.96$) 18, 0.005	45 40 35 30 25 0[a]
Olin 101 elutriate	Mortality CT	100%	Not performed on two samples		
Olin 101	Mortality ST	10.23% (9.56%–10.94%)	7.48	6 ($F = 5.26$) 14, 0.005)	Not separable
Morton Thiokol	Sublethal GACVT		3.45	20 ($F = 3.06$) 24, 0.005	50 35 25 15 0[a]
Olin 101 5%[a]	Sublethal GACVT		9.75	4 ($F = 4.79$) 25, 0.005	48 6 26 20 0[b]
Olin 101 10%[a]	Sublethal GACVT		7.02	4 ($F = 4.98$) 40, 0.005	32 24 18 6 0[b]
Olin 101 20%[a]	Sublethal GACVT		32.31	3 ($F = 6.03$) 18, 0.005	18 12 6 0[b]

Note: Any two means not underscored by the same line are significantly different. Any two means underscored by the same line are not significantly different

[a] Levels tested shown as a percentage of site soil by weight
[b] Hours after the start of the test

samples or elutriates for detection of water-soluble toxicants. The CT has certain advantages in that it is a short test (48-h) and has been shown to be highly reproducible (Edwards 1983). However, for environmental samples, based on these results its use is limited to detection of water-soluble toxicants. Negative results for the CT, however, should not be interpreted to mean that the parent soil mixture itself is not toxic to soil invertebrates.

The Olin Chemical Co. sample had a relatively higher toxicity in the soil matrix than the elutriate as indicated by the ST LC_{50} (Table 2). The Olin Chemical Co. soil sample was more toxic than the Morton Thiokol sample, with an estimated LC_{50} of 10.23% site soil. The Olin sample was found to have approximately 6800 ppm elemental sulfur, which is believed to be responsible for the low pH (Table 1). The Olin sample pH (Table 1) may have contributed to the overall toxicity of the sample. However, the mixtures of artificial soil and Olin soil upon examination show pH values within the range of other samples processed in this laboratory; yet some of these other samples were nontoxic to earthworms. An examination of 28 sample pH values and LC_{50} estimates show a correlation of 0.28, which indicates that pH is not the controlling factor producing toxicities.

Although there was a significant difference in the percentage mortality for the Olin sample as assessed by the ANOVA *F* test (i.e., the null hypothesis was rejected), none of the contrasts of all possible contrasts (e.g., u1–u2, u2–u3) differ significantly from 0.0 (Table 2). This result is uncommon, but one of the possible outcomes of multiple comparisons (Neter and Wasserman 1974).

The ST appears to be more appropriate than the CT using water leachates for measuring the potential soil toxicity of environmental samples which contain unknown chemicals at unknown concentrations. Heimbach (1984) compared the ST and the CT in the laboratory, showing a lack of high correlation for the same chemicals. His results compare favorably with ours, indicating that the mechanism of uptake as well as a difference in exposure time may be the most important differences between these two tests. In our tests of environmental samples many potential toxicants may not be extracted by water elutriation.

Sublethal effects of Morton Thiokol soil using GACV measurements were made on specimens using the ST protocol (Table 2). The first significant difference compared with the control was at the 35% level.

All three concentrations of the Olin Chemical Co. soil sample showed a significant *F* ratio for GACV at various time intervals (Table 2). At 5%, the GACV reduction seemed to occur rapidly and then level off (Fig. 1). For the 10% level there was a gradual

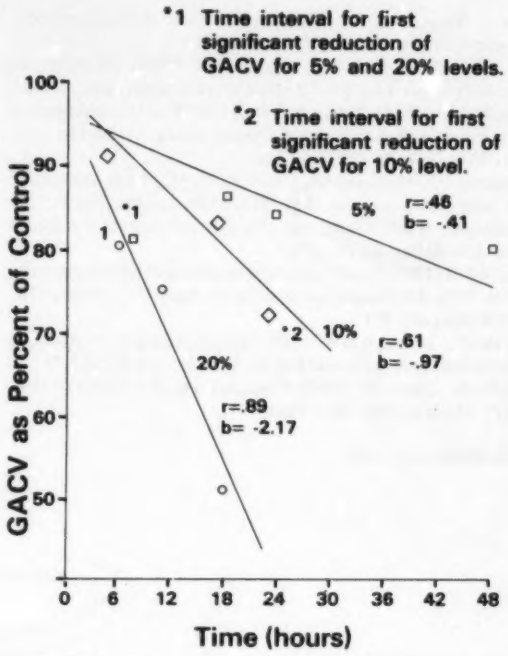

Fig. 1. Relative Giant Axon Conduction Velocity (GACV) plotted against time for Olin Chemical Co. soil at three concentrations, 5%, 10%, and 20% (site soil/artificial soil; dry wt./dry wt). Lines were drawn by regression

reduction in GACV with time, with the first significant reduction in GACV at 24 h. This is considerably different from both the other levels and may represent a difference in exposure, perhaps through reduced feeding or locomotor activity. The 20% level response for GACV showed a very quick reduction in conduction velocity, being significant after 6 h as was seen at the 5% level (Table 2).

The GACVT is suggested as a companion test with the ST as a means of making a rapid assessment of neurotoxic effects. Two important results of the GACVT evaluation in this study are: (1) this technique can detect neurotoxins in complex mixtures (environmental samples) as well as single compounds and (2) this technique can detect toxicities in shorter periods of exposure at levels well below toxicity levels that produce mortalities. The ST mortality test was equally sensitive when compared with the GACV sublethal test for the Morton Thiokol samples where the lowest level of significant GACV reduction was approximately in the same area, 25%–35%, as that of the mortality end point, 25%–30%. Although the ST mortality test results for the Olin Chemical Co. sample could not be separated by multiple comparisons analysis (Table 2) when the LC_{50} was approximately 10% of the site soil, the sublethal GACVT for this sample was significant after 6 h at 5% of site soil. The GACVT end points were at least five times more sensitive than the CT and the ST results for the Olin samples.

Laboratory tests of this type can detect acute (ST and CT) and sublethal (GACVT) effects, but they can only infer a similar impact for field populations (Moriarty 1983). Therefore, measurement of individual and population effects should be made in well-designed field experiments whenever possible (Haque and Pflugmacher 1985).

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References

Callahan CA (in press) Earthworms as ecotoxicological assessment tools. In: Edwards CA, Neuhauser EF (eds) Proc. Int. Conf. on Earthworms in Waste and Environmental Management. 23–27 July, 1984, Cambridge, England

Dean-Ross D (1983) Methods for the assessment of the toxicity of environmental chemicals to earthworms. Reguly Tox Pharm 3:48–60

Drewes CD, Vining EP (1984) In vivo neurotoxic effects of dieldrin on giant nerve fibers and escape reflex function in the earthworm, *Eisenia foetida*. Pest Biochem Phys 22:93–103

Drewes CD, Vining EP, Callahan CA (1984) Non-invasive electrophysiological monitoring: a sensitive method for detecting sublethal neurotoxicity in earthworms. Env Tox Chem 3-:599–607

Drewes CD, Vining EP, Callahan CA (in press) Electrophysiological detection of sublethhal neurotoxic effects in intact earthworms. In: Edwards CA, Neuhauser EF (eds) Int. Conf. on Earthworms in Waste and Environmental Management. 23–27 July 1984, Cambridge, England

Ebing W, Pflugmacher J, Haque A (1984) Der Regenwurm als Schlüsselorganismus zu Messung der Bodenbelastung mit organischen Fremdchemikalien. Ber über Landwirtschaft 62-:222–255

Edwards CA (1983) Report of the second stage in development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms. Report to the Commission of the European Communities, Rothamsted Experimental Station, Harpenden, Herts, U.K.

European Economic Community EEC Directive (1982) Methods for the determination of ecotoxicity level1.DG X1/127/82, Rev. 1, No. 79/831, annex V, part C. Commission of the European Community

Fisher SW (1984) A comparison of standardized methods for measuring biological activity of pesticides to the earthworm, *Lumbricus terrestris*. Ecotox Environ Safety 8:564–571

Goats G, Edwards CA (1982) Testing the toxicity of industrial chemicals to earthworms. Rothamsted Reports 104–105

Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Env Sci Tox 11:714–717. Correction: ibid 12:417 (1978)

Haque A, Ebing W (1983) Toxicity determination of pesticides to earthworm in the soil substrate. 2 Pflkrankh Pflschutz 90:397–401

Haque V, Pflugmacher J (1985) Einflüsse von Pflanzenschutzmitteln auf Regenwürmer. Ber über Landwirtschaft 198 :176–189

Heimbach F (1984) Correlations between three methods for determining the toxicity of chemicals to earthworm. Pest Sci 15:605–611

Karnak RE, Hamelink JL (1982) A standardized method for determining the acute toxicity of chemicals to the earthworm. Ecotox Environ Safety 6:216–222

Kramer CY (1956) Extension of multiple range tests to group means with unequal numbers of replications. Biometr 12:307–310

Malecki MR, Neuhauser EF, Loehr RC (1982) The effects of metals on the growth and reproduction of *Eisenia fetida* (Oligochaeta, Lumbricidae). Pediobiologia 24:129–137

Moriarty F (1983) Ecotoxicology. Academic, New York

Neter J, Wasserman W (1974) Applied linear statistical models. Richard D. Irving, Inc., Homewood, IL

Neuhauser EF, Loehr RC, Malecki MR (1985). Methods for evaluating the biological impact of potentially toxic wastes applied to soils. In: Petros K, Lacy J (eds) Fourth symposium of hazardous and industrial solid waste testing. Arlington, VA. 2–4 May 1984. ASTM Phil., PA

Neuhauser EF, Malecki MR, Loehr RC (1982) Land application of industrial wastes. In: Bell JM (ed) Proc of the 37th Purdue Industrial Waste Conference, Ann Arbor Science Publishers Inc Ann Arbor, pp 253–258

Porcella DB (1983) Protocol for bioassessment of hazardous waste sites. U.S. Environmental Protection Agency Corvallis, OR. EPA 600/2–83–054

Roberts BL, Dorough HW (1984) Relative toxicities of chemicals to the earthworm *Eisenia foetida*. Env Tox Chem 3:67–78

Steel RGD, Torrie JH (1960) Principles and procedures of statistics. McGraw-Hill, New York

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